JOURNAL OF BACTERIOLOGY, Dec. 2001, p. 7341–7353 Vol. 183, No. 24
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.24.7341–7353.2001

Transcription Profiling-Based Identification of *Staphylococcus aureus* Genes Regulated by the *agr* and/or *sarA* Loci

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Received 2 July 2001/Accepted 26 September 2001

The advent of transcription profiling technologies has provided researchers with an unprecedented ability to study biological processes. Accordingly, a custom-made Affymetrix GeneChip, constituting >86% of the *Staphylococcus aureus* genome, was used to identify open reading frames that are regulated by *agr* and/or SarA, the two best-studied regulators of the organism's virulence response. RNA extracted from wild-type cells and *agr*, *sarA*, and *agr sarA* mutant cells in the early-, mid-, and late-log and stationary phases of growth was analyzed. Open reading frames with transcription patterns expected of genes either up- or downregulated in an *agr*- and/or SarA-dependent manner were identified. Oligonucleotide microarray and Northern blot analyses confirmed that the transcription of several known virulence genes, including *hla* (alpha-toxin) and *spa* (protein A), is regulated by each effector and provided insights about the regulatory cascades involved in both alpha-hemolysin and protein A expression. Several putative virulence factors were also identified as regulated by *agr* and/or SarA. In addition, genes that are involved in several biological processes but which are difficult to reconcile as playing a direct role in the organism's pathogenesis also appeared to be regulated by each effector, suggesting that products of both the *agr* and the *sarA* locus are more-global transcription regulators than previously realized.

Staphylococcus aureus is a major cause of human disease. The organism causes a variety of clinical manifestations, ranging from localized skin infections to severe sepsis, and is a leading cause of hospital-acquired infection (3). Despite advances in antibacterial chemotherapy, *S. aureus* strains have demonstrated resistance to all currently available antibiotics. Due in part to the immense clinical importance of this organism, an enormous amount of effort has been directed toward identifying the genes and regulatory mechanisms associated with *S. aureus* pathogenesis. Collectively, this work has demonstrated that the organism's pathogenesis can be attributed to its capacity to produce a variety of virulence factors (29).

The identification of virulence factors and the regulatory networks that influence their expression has been facilitated by the observation(s) that many, if not most, virulence genes are expressed in laboratory cultures. While there is currently a substantial list of staphylococcal virulence factors, it is likely that this list is incomplete and is skewed by the limitations of the experiments used to identify them. Virulence factors that have already been identified generally include (i) bacterial surface proteins that are involved in processes such as adhesion and evasion of the host immune response and (ii) secreted exoproteins that degrade host tissue(s) and inactivate host defensive mechanisms (29).

The genes encoding most virulence factors belong to an extensive regulon that is coordinately regulated in response to a variety of intra- and extracellular signals (1, 5, 21). Octapeptide signaling molecules that are produced as laboratory cultures increase in cell density are the best-studied "stimuli" of the virulence response, mediating a transition in expression of the genes encoding virulence determinants from a predominance of cell surface-expressed genes to a predominance of genes encoding exoproteins (26).

The density-dependent regulation of most virulence factors is mediated by regulatory loci such as the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sarA*) loci (9, 19). In the laboratory setting, post-exponential growth activates the *agr* locus, which contains two divergent promoters, P2 and P3, that direct expression of RNAII and RNAIII transcripts, respectively (19). RNAII encodes four proteins, AgrB, AgrD, AgrC, and AgrA, all of which are required for *agr*-mediated virulence factor regulation (19, 27). AgrD and AgrB act to generate an octapeptide quorum-sensing molecule (autoinducing peptide [AIP]), which, after reaching an extracellular threshold concentration, stimulates activation of AgrC and AgrA, the sensor and regulator, respectively, of a two-component regulatory pathway (17–19, 27, 28). Activated AgrA results in the upregulation of RNAII and RNAIII production; the latter is the effector molecule of the *agr* response (25, 27, 28). RNAIII expression is, in part, responsible for the upregulation of exoprotein production and the downregulation of cell surface protein transcription during the late-log and stationary phases of growth. The manner in which RNAIII

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TABLE 1. *S. aureus* strains used in this study

Strain	Relevant genotype	Source or reference
RN27	*agr*$^+$ *sarA*$^+$	
RN6911	*agr sarA*$^+$; RN27 derivative with an *agr*::*tetM* mutation	28
ALC488	*agr*$^+$ *sarA*; RN27 derivative with a *sarA*::*ermC* mutation	8
ALC842	*agr sarA*; *agr* mutation in RN6911 transduced into ALC488	8

regulates target genes is not yet understood, but it is clear that the molecule acts mainly at the level of transcription. RNAIII also encodes the structural gene for delta-hemolysin, although the transcript, rather than the protein product, acts as a global virulence factor regulator (28).

The *S. aureus* accessory regulatory (SarA) protein also influences both exoprotein and cell surface protein expression (9). The *sarA* locus contains three overlapping transcripts designated *sarA*, *sarC*, and *sarB*, each of which has a common 3′ end encoding SarA (2). SarA binds to conserved regions termed Sar boxes within promoter regions of genes encoding cell surface proteins (*spa*, encoding protein A), genes encoding exoproteins (*hla*, encoding alpha-hemolysin), and *agr* (12). SarA binding to *agr* promoter elements augments both RNAII and RNAIII transcription and therefore contributes to virulence factor regulation indirectly (7, 13). SarA has also been shown to regulate expression of *spa* and *hla* in an *agr* mutant background, indicating that SarA controls regulation of certain virulence factors directly, in an *agr*-independent manner.

Because RNAIII and/or SarA influences the transcription of most known virulence factors, it was hypothesized that additional, previously uncharacterized potential virulence factors may be identified by analyzing the *S. aureus* genome for open reading frames (ORFs) that are expressed in an *agr*- and/or SarA-dependent manner. Furthermore, such an analysis may begin to unravel the overlapping RNAIII and SarA regulatory contributions to known virulence factors. Using an *S. aureus* GeneChip (Affymetrix), we have identified genes that produce transcript patterns expected of genes regulated by the *S. aureus* *agr* and/or *sarA* locus. In addition to confirming the predicted expression patterns of a number of known virulence factors, and thus validating the methodology, we identified a set of putative virulence factors as being regulated by each effector. Moreover, expression patterns of genes involved in a number of biological processes that are difficult to reconcile as contributing directly to *S. aureus* pathogenesis produced expression profiles expected of genes regulated in an *agr*- and/or SarA-dependent, density-dependent manner. These results suggest that, in addition to regulating virulence factors, each effector is a more-general transcriptional regulator than previously recognized.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *S. aureus* strains used in this study are listed in Table 1. Strains were grown overnight in 25 ml of brain heart infusion (BHI) medium at 37°C with aeration. Overnight cultures were used to inoculate (1:100 dilution) 1.5 liters of fresh BHI medium. Cultures were incubated with vigorous aeration at 37°C and aliquots were removed at the indicated growth phases as determined by growth phase analysis (data not shown). Cells from each aliquot were pelleted by centrifugation at 5,000 × *g* for 10 min at 4°C in a Beckman JA-10 rotor and were stored at −80°C.

RNA extraction. Total bacterial RNA was extracted from samples by resuspending each cell pellet at a concentration of 10^9 CFU per ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 50 μg of lysostaphin (Amicon)/ml. Resuspensions were incubated at 37°C for 60 min, and 1×10^{10} to 5×10^{10} CFU was then applied to a Qiagen RNeasy Maxi column, according to the manufacturer's recommendations for prokaryotic RNA isolation. RNA concentrations were determined by spectrophotometry (an optical density at 260 nm of 1.0 equals 40 μg of RNA/ml). To assess RNA integrity and for Northern blot analysis, 2 μg of each RNA sample was electrophoresed in a 1.2% agarose–0.66 M formaldehyde gel, according to Qiagen RNA electrophoresis recommendations.

Northern blot analysis. RNA samples were transferred from formaldehyde-containing agarose gels to Biodyne B membranes (Gibco-BRL) by standard molecular procedures. High-stringency hybridization was performed in Kapak/Scotchpak heat-sealable pouches containing 12 ml of PerfectHyb Plus hybridization buffer (Sigma) and 10 μl of radiolabeled randomly primed DNA synthesis products from PCR-generated templates at 42°C. Membranes were subjected to high-stringency washes and were analyzed by phosphorimaging (Bio-Rad Phosphorimager) or densitometry (Bio-Rad). The following PCR primers were used to generate PCR templates: for 16S rRNA (478 nucleotides [nt] of GenBank accession no. Y15856), 5′-AAATCTTGACATCCTTTGACAACTC-3′ and 5′-CTAGCTCCTAAAAGGTTACTCCACC-3′; for *spa* (1,999 nt of GenBank accession no. M18264), 5′-AAGATTTAATTGAAACAATCCACCA-3′ and 5′-GACCAGGTTTGATCATGTTTTTATC-3′; for *hla* (769 nt of GenBank accession no. X01645), 5′-TAGGTTCCATATTGATGAATCCTGT-3′ and 5′-ATATTTGTTTGTTGTTTGGATGCTT-3′; and for RNAIII (480 nt of GenBank accession no. AF288215), 5′-GGGGCTCACGACCATACTTA-3′ and 5′-GGAGTGATTTCAATGGCACA-3′. All PCR products were subjected to restriction enzyme analysis and were gel purified prior to labeling reactions.

mRNA enrichment, fragmentation, and biotinylation. For mRNA enrichment reactions (240 μl), 200 μg of total bacterial RNA was mixed with 0.7 μM (final concentration) rRNA-specific oligonucleotide mix (5′-GATACGGCTACCTTGTT-3′, 5′-TCAACCTTGCGGTCGTACTC-3′, 5′-TCCGGATAACGCTTGCCACC-3′, 5′-AGCACTTATCCCGTCCACAC-3′, 5′-CTACAGTAAAGCTCCACGGG-3′, and 5′-TCCCCATCACAGCTCAGCCT-3′). Aliquots (30 μl) were transferred to 0.5-ml thin-walled Eppendorf tubes (Perkin-Elmer), and solutions were incubated at 70°C for 5 min in a thermocycler (Perkin-Elmer). Reverse transcriptase reaction mixture (50 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol, 0.5 mM each deoxynucleoside triphosphate, 735 U of RNAguard [Amersham Pharmacia Biotech], and 3,000 U of Moloney murine leukemia virus reverse transcriptase [Epicentre]) was then added. Solutions were incubated at 42°C for 25 min, followed by 45°C for 20 min. For rRNA removal, 0.5 U of RNase H (Epicentre) was added and mixtures were incubated first at 37°C for 45 min and then at 65°C for 5 min. DNase I (0.12 U/μl; Amersham Pharmacia Biotech) and 0.225 U of RNAguard were added, and mixtures were incubated at 37°C for 20 min. Reactions were terminated by addition of 10 mM EDTA. Samples were applied to a Qiagen RNeasy minicolumn, according to manufacturer recommendations for RNA clean-up. For RNA fragmentation, 20 μg of an mRNA-enriched sample was mixed with fragmentation buffer (1× T4 polynucleotide kinase buffer [New England Biolabs], 70 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 5 mM dithiothreitol) and incubated at 95°C for 30 min and then cooled to 4°C. Samples were then 5′-thiolated by addition of 0.1 mM γ-S-ATP and 1 U of T4 polynucleotide kinase (New England Biolabs) and were incubated at 37°C for 50 min, followed by 65°C for 10 min. Samples were then ethanol precipitated and subsequently biotinylated by addition of 2 mM polyethylene oxide (PEO)-iodoacetyl-biotin and 30 mM morpholinepropanesulfonic acid (MOPS), pH 7.5. Unincorporated biotin molecules were removed by passing RNA mixtures through a Qiagen RNA/DNA minicolumn according to manufacturer recommendations.

***S. aureus* GeneChip design.** Preliminary genomic sequence data of the *S. aureus* COL strain were obtained from The Institute for Genomic Research (TIGR) website (http://www.tigr.org). The sequence consisted of approximately 2,000 contigs, which were concatenated into a single sequence alternating with an 18-nt sequence containing stop codons in all 6 frames. ORF predictions were made using a combination of GLIMMER 1.0 (32) and GeneMark.hmm (23), with a minimum ORF size of 75 nt. In all, 4,528 ORFs, 12 tRNAs, and 3 rRNAs were tiled, with an average of 25 probe sets per ORF. Due to the preliminary state of the genome sequence, many genes are represented on the chip as partial, duplicate, or overlapping fragments. Recent analyses based on the updated sequences of the genomes of COL, NCTC 8325 (OU-ACGT), and MRSA (Sanger Centre) suggest that these 4,528 tilings represent approximately 2,700 to

2,900 individual genes and that >86% of the *S. aureus* COL genome is represented on the chip.

GeneChip hybridization and washing. Prior to RNA hybridization, *S. aureus* GeneChips (Affymetrix) were brought to room temperature, washed once with hybridization buffer (100 mM *N*-morpholinoethanesulfonic acid [MES], 1 M Na^+, 20 mM EDTA, 0.01% Tween 20), loaded (250 μl) with hybridization buffer, and incubated at 45°C for at least 10 min. Hybridization mixture (1.5 μg of biotinylated mRNA-enriched RNA, 50 pM oligonucleotide B2 [Affymetrix], 0.1 mg of herring sperm DNA/ml, 0.5 mg of acetylated bovine serum albumin [BSA; Gibco-BRL], and 1× control spike-in cocktail [Genetics Institute] in 1× hybridization buffer) was denatured by incubating at 95°C for 5 min, followed by 45°C for 5 min, and was centrifuged at high speed on a tabletop microcentrifuge for 20 min at room temperature to pellet all nonsolubilized material. A 200-μl volume of hybridization mixture was then loaded onto a GeneChip and incubated for 15 h at 45°C. Following hybridization, the RNA-containing mixture was removed and stored at −80°C. GeneChips were then washed 20 times with nonstringent buffer (6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 0.01% Tween 20, 0.005% antifoam) at 25°C. Chips were then subjected to 60 washes with stringent buffer (100 mM MES, 0.1 M Na^+, 0.01% Tween 20) at 45°C. Following washing, the hybridized RNA was treated with primary stain (10 μg of streptavidin, 2 mg of acetylated BSA) in stain buffer (100 mM MES, 1 M Na^+, 0.05% Tween 20, 0.005% antifoam) for 10 min at 25°C and was washed 40 times with nonstringent buffer. Next each GeneChip was subjected to a secondary stain containing 2 mg of acetylated BSA, 0.1 mg of goat immunoglobulin G, and 5 μg of biotinylated anti-streptavidin (Vector Laboratories) in stain buffer for 10 min at 25°C. The chip-bound RNA molecules were labeled with 10 μg of streptavidin-phycoerythrin conjugate (Molecular Probes)/ml in stain buffer for 10 min at 25°C. Excess label was removed by 100 nonstringent washes at 25°C.

GeneChip analysis. To increase reproducibility, each experiment was performed in duplicate (RNA extraction from various phases of growth for each strain) and each RNA sample was hybridized to two separate GeneChips. The work here constitutes a total of 16 GeneChips for each strain analyzed. Each GeneChip was scanned at 570-nm, 3-μm resolution in an Affymetrix GeneChip scanner. Affymetrix algorithms calculated signal intensities (average difference) and made present or absent determinations for each gene, as previously described (22). Next, to normalize for global systematic variations that could be caused by inconsistencies in loading, each average difference value was divided by the median average difference for a given GeneChip. Normalized intensity values were then averaged for each gene at each growth phase. GeneSpring, version 3.2.11, software (Silicon Genetics, Redwood City, Calif.) was used to plot normalized intensity values across growth phases. GeneSpring algorithms also determined signal strength values for each gene within a strain as the product of the average normalized signal intensity value of the gene of interest and the median normalized signal intensity of the gene taken throughout growth phases. To identify genes that are below the detection limit of the system, the signal strengths indicative of genes with profiles at the level of noise were determined for each strain as the average signal strength of genes considered absent (via GeneChip algorithms) plus 2 standard deviations.

Identification of Sar boxes. Intergenic regions were identified within the *S. aureus* N315 genome by comparing ORF nucleotide coordinates (20). Putative Sar boxes (12) were identified using the Genetics Computer Group (Madison, Wis.) program (Wisconsin Sequencing Package).

RESULTS

Identification of *S. aureus* virulence factors. In the laboratory setting, expression of many *S. aureus* virulence factors follows a predictable pattern. Cell surface virulence factors, such as protein A (encoded by *spa*), are predominately expressed during early-log-phase growth, but as the density of a growing culture increases, transcription of these genes decreases. Genes encoding extracellular virulence factors, such as alpha-toxin (encoded by *hla*), demonstrate a reciprocal phenotype; they tend to be transcribed at basal levels during early-log-phase growth and are upregulated at higher cell densities. This density-dependent regulation of most virulence determinants can be controlled by a product of the *agr* locus, RNAIII, and/or SarA.

Although there is currently a sizeable list of RNAIII- and SarA-regulated putative virulence factors, it seems likely that this list is incomplete and that an expansion of known virulence factors is needed to better understand *S. aureus* pathogenic processes. In an effort to identify previously unrecognized *agr*- and/or SarA-regulated genes, we performed transcription profiling on RNA samples extracted from wild-type cells (RN27) and *agr* (RN6911), *sarA* (ALC488), and *agr sarA* (ALC842) mutant cells during various phases of growth (early-, mid-, and late-log and stationary phases).

***agr* regulation by SarA.** To validate the transcription profiling methodology used, we first investigated whether the *agr* locus transcripts, RNAII and RNAIII, produced expression patterns mimicking previously reported data. It is well established that *agr* is temporally regulated in a growth phase-dependent manner. Basal levels of RNAII and RNAIII are detected during early-log-phase growth; transcription subsequently increases as cells progress through growth phases (16, 34). Additionally, Cheung and colleagues have shown that SarA interacts with *agr* promoter regions and facilitates RNAII and RNAIII transcription (7, 15).

As expected, transcription profiling of RNA extracted from cells at various phases of growth demonstrated that both RNAII and RNAIII production increased (4.8- and 5.7-fold, respectively) as wild-type cells (RN27) transitioned from the early-log to stationary phase of growth, with maximal expression detected during post-exponential growth (Fig. 1A and B). In contrast, both RNAII and RNAIII transcript levels were below the detection limits of the system in *agr* (RN6911) and *agr sarA* (ALC842) mutant cells (Fig. 1A and B). Moreover, Affymetrix GeneChip software analysis (see Materials and Methods) of RNA samples from RN6911 and ALC842 cultures determined that the genes constituting RNAII (*agrA*, *agrB*, *agrC*, and *agrD*) and RNAIII transcripts were absent in all phases of growth (data not shown). As shown in Fig. 2A, these results correlate well with those obtained by Northern analysis, which indicated that RNAIII was induced 14.2-fold in RN27 cells during post-exponential growth but was not detectable in RN6911 cells. Furthermore, our transcription profiling data (Fig. 1B) demonstrated a ~2.6-fold decrease in RNAIII production by a *sarA* mutant strain (ALC488) during stationary-phase growth, in comparison to wild-type transcript levels, confirming that SarA is required for wild-type levels of *agr* transcription. Collectively these results indicate that our transcription profiling data are in good agreement with previous observations and confirm that both RNAII and RNAIII are expressed in a cell density-dependent manner.

***agr* and SarA regulation of alpha-toxin.** To further validate the methodology and extend our knowledge about the transcription of the well-studied *agr*-regulated exoprotein alpha-toxin (encoded by *hla*), we investigated whether our transcript profiles correlated well with published *hla* expression patterns. It has been shown that RNAIII production promotes *hla* expression, with maximum levels being reached at the post-exponential phase of growth (6, 34). As shown in Fig. 1C, transcription profiling results demonstrated a 39-fold increase in *hla* transcription in wild-type (RN27) cells as they transitioned from the early-log to stationary phase of growth, and these results were in good agreement with the 20.9-fold increase seen in our Northern analysis (Fig. 2B). In contrast, the increase in transcription was reduced to 2.2-fold in RN6911 cells

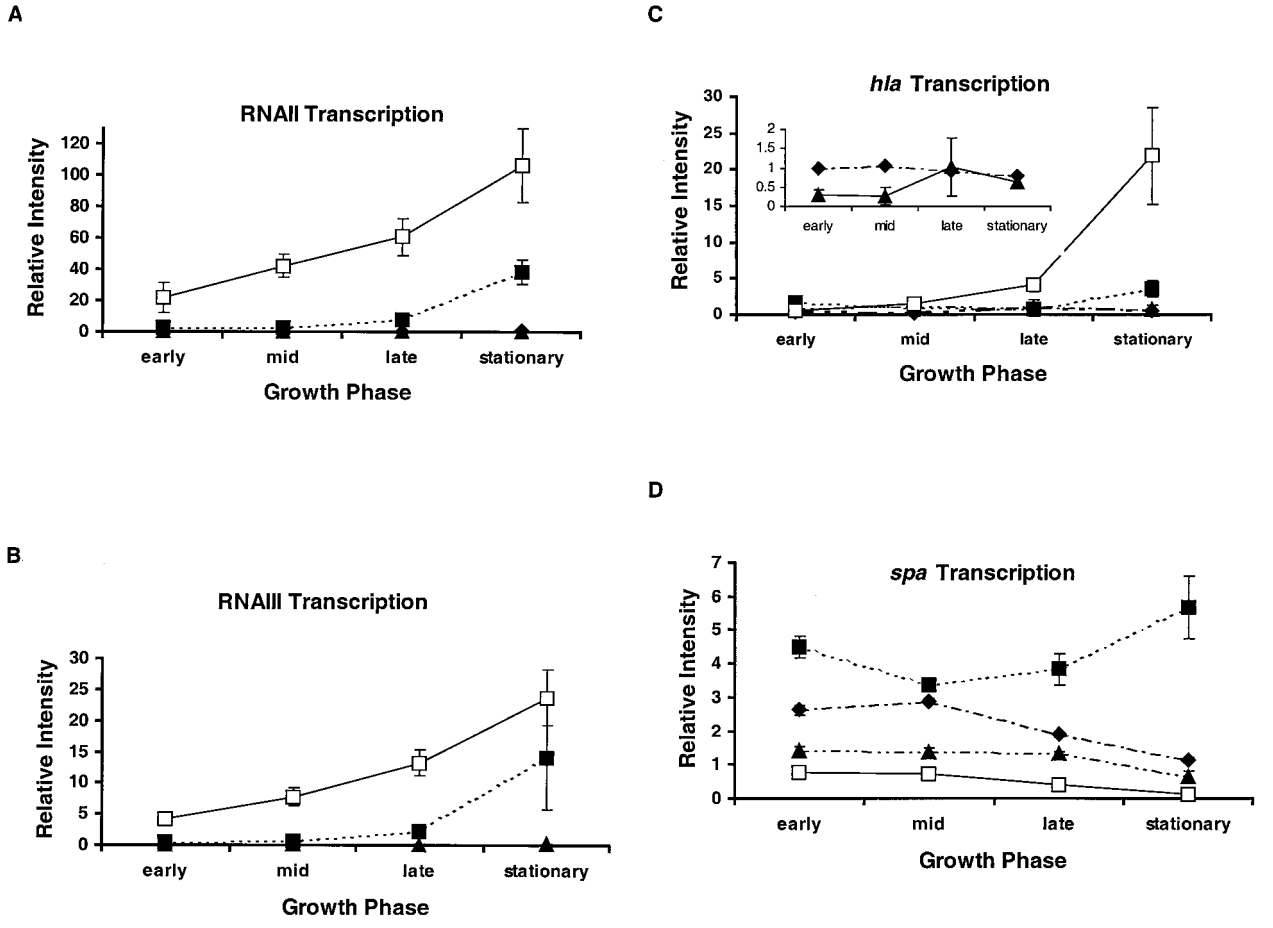

FIG. 1. Transcription profiles of known RNAIII and SarA-regulated genes. Transcript levels for wild-type (□), *agr* mutant (▲), *sarA* mutant (■), and *agr sarA* double-mutant (◆) cells were measured during the early-, mid-, and late-log and stationary phases of growth (*x* axis). Data points were plotted as relative intensity values (*y* axis) (as described in Materials and Methods). (A) Average signal intensities for genes constituting RNAII transcripts (*agrB*, *agrD*, *agrC*, and *agrA*). (B) Signal intensities of RNAIII transcripts. (C) Profiles of alpha-toxin (*hla*) transcript titers. (Inset) *agr* and *agr sarA* mutant results. (D) Profiles of protein A (*spa*) transcript titers.

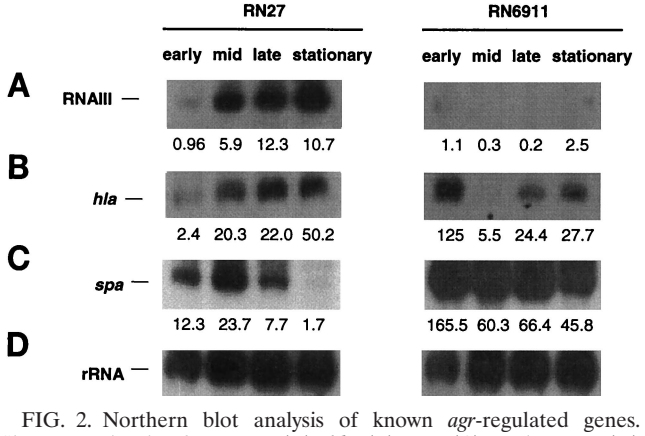

FIG. 2. Northern blot analysis of known *agr*-regulated genes. Shown are levels of RNAIII (A), *hla* (B), *spa* (C), and rRNA (D) transcripts during the early-, mid-, and late-log and stationary phases of growth of wild-type (RN27) or *agr* mutant (RN6911) cells. Signal intensities were determined by densitometry and normalized to rRNA signals. Normalized relative signal intensities are shown below each panel.

(Fig. 1C), confirming that a product of the *agr* locus mediates *hla* upregulation during the post-exponential phase. These results also indicate that an additional regulatory mechanism(s) may account for the slight (2.2-fold) increase in *hla* expression in *agr* mutant cells. Indeed, SarA binding sites have been identified within the *hla* promoter region, and SarA has been shown to bind directly to the promoter region and induce *hla* expression in an *agr*-independent manner (6, 12). To determine whether this modest increase in expression could be attributed to SarA, as opposed to an additional virulence regulator(s), the profiles of *hla* transcripts were compared for various growth phases of *sarA* and *agr sarA* mutant cells. As expected, *hla* expression was marginally induced in post-exponential *sarA* mutant cells (Fig. 1C). However, because SarA is necessary for complete expression of *agr*, the strain is effectively *agr* deficient and the observed reduction in *hla* expression may be a direct consequence of decreased production of RNAIII (10, 12). Yet *hla* was constitutively transcribed during various phases of growth in *agr sarA* mutant (ALC842) cells (Fig. 1C), confirming that SarA both directly and indirectly regulates alpha-hemolysin transcription. Interestingly, GeneChip analysis (see Materials and Methods) determined that *hla*

transcripts were present in all phases of ALC842 growth (data not shown), implying that neither regulator is required for basal levels of alpha-hemolysin transcription. In addition, these results suggest that both RNAIII and SarA are required for wild-type levels of *hla* transcription during post-exponential aerobic growth.

***agr* and SarA regulation of protein A.** It has previously been shown that wild-type *S. aureus* transcribes *spa* during early-log-phase growth and that its transcription is reduced when RNAIII is produced. Conversely, *spa* is transcribed at a high level throughout all phases of growth in an *agr*-null strain (34). The *spa* promoter contains SarA boxes (12). Both Northern analysis and *spa*-reporter fusion studies have shown that SarA is a repressor of protein A transcription (4, 10). To determine whether transcription profiling could identify genes known to be downregulated by each effector, we compared the protein A (*spa*) transcript profiles of each strain. As shown in Fig. 1D, wild-type cells maximally expressed *spa* at early- and mid-log phases of growth, and transcription decreased 6.8-fold as cells entered stationary phase. A similar phenotype (a 7.2-fold decrease) was observed by Northern analysis (Fig. 2C). Comparison of signal strengths (see Materials and Methods) demonstrated that *agr* mutant cells expressed high levels of *spa* throughout all phases of growth (average normalized signal strength value, 1,031.5, as opposed to 170.8 in wild-type cells), confirming that the *agr* locus is a potent repressor (>5-fold) of *spa* transcription. Northern blot analysis (Fig. 2C) confirmed those observations. Transcription profile analysis also revealed that, like *agr* mutant cells, *spa* transcription is significantly increased, across all growth phases, in the *sarA* mutant (Fig. 1D). This finding is in good agreement with previously published *spa* expression levels in *sarA* mutant cells (8).

***agr*-stimulated ORF identification.** Given the level of correlation between the transcription profiling data obtained in this study and previously reported results for RNAII, RNAIII, *hla*, and *spa* expression, we felt comfortable searching for genes that were previously unrecognized as being regulated by the *agr* and/or *sarA* locus. To identify genes upregulated by a product of the *agr* locus, we took advantage of the observation that transcription of most identified *agr*-upregulated virulence genes, such as *hla*, increases with cell density in wild-type cells but does not increase in a density-dependent manner in *agr* mutant cells. Using GeneSpring software, we were able to compare transcript profiles of genes by analyzing RNA samples taken during various phases of growth from wild-type cells and *agr* (RN6911), *sarA* (ALC488), and *agr sarA* (ALC842) mutant cells. Genes demonstrating transcript patterns expected of those upregulated in an RNAIII-dependent manner were identified by querying for genes that were upregulated at least 2- and 1.5-fold as wild-type and ALC488 cells, respectively, moved from the early-log to the stationary phase of growth but were not upregulated 2-fold or were expressed at background levels in RN6911 and ALC842 cells. Alternatively, genes that were constitutively expressed in wild-type cells but were expressed at twofold-lower levels in both RN6911 and ALC842 cells were likely to be stimulated by a product of the *agr* locus. In each case the gene also had to be determined to be present in at least one wild-type stationary-phase sample, according to Affymetrix GeneChip software restrictions (Table 2). As expected, genes constituting the *agr* RNAII transcript (*agrB*, *agrD*, *agrC*, and *agrA*) produced the looked-for transcript profiles. Likewise, RNAIII (*hld*, encoding delta-hemolysin) appeared to be induced in an *agr*-dependent manner. Several known RNAIII-inducible genes were also identified, including *splA, splB, splD*, and *splF* (encoding serine protease) and *hla* (encoding alpha-toxin) (30, 31). Other extracellular virulence factors, such as *hlgB* and *hlgC* (encoding gamma-hemolysin) and *geh* (encoding lipase), were also identified.

Identification of *agr*-repressed ORFs. Table 3 lists 64 genes that produced transcript patterns expected of genes downregulated in an *agr*-dependent manner. These genes appeared to be transcribed at levels at least twofold higher in stationary-phase *agr* mutant cells than wild-type cells. Additionally, genes were considered present in at least one wild-type stationary-phase sample, according to GeneChip software restrictions. As expected, known RNAIII-downregulated genes, such as *spa* (34), were detected. Several additional cell wall-associated proteins, such as *dlt* (involved in teichoic acid linkage to cell wall and resistance to defensins), *isaA* (cell wall secretory protein), and *mnhA*, *mnhF*, and *mnhG* (constituting a heterologous surface receptor) are potentially repressed by *agr*.

Effects of SarA on *agr*-regulated genes. As shown in Fig. 1A and B, SarA is required for wild-type levels of *agr* transcription. More specifically, RNAII and RNAIII transcripts were decreased ~2.6-fold (±0.6-fold) in *sarA* mutant (ALC488) cells. Extending this observation, we identified potential *agr*-stimulated genes (Table 2) that demonstrate a similar fold reduction (within 2 standard deviations) in ALC488 cells. Genes meeting this criterion are presumed to be indirectly regulated by SarA and are indicated in Table 2. Likewise, genes suspected to be downregulated by *agr* (Table 3) that demonstrated a similar fold increase in transcript levels in ALC488 cells may be indirectly regulated by SarA, as indicated in Table 3. However, it should be kept in mind that RNAIII production has different regulatory effects on responsive genes (i.e., *hla* is dramatically upregulated in the presence of RNAIII, whereas expression of other genes, such as *tst* [encoding toxic shock syndrome toxin 1], is stimulated to a lesser extent) (26). Therefore, caution should be exercised in further interpreting these results. Yet comparison of genes determined to be directly regulated by SarA (described below) is in good agreement with our indirect SarA analysis.

Identification of SarA-upregulated genes. A series of transcription profile comparisons were performed to identify genes expected to be upregulated by SarA in a density-dependent manner. First, genes upregulated at least twofold as wild-type cells transition from early-log to stationary phase were identified. Additionally, transcripts had to be considered present within wild-type stationary-phase samples by Affymetrix GeneChip analysis. From this list, genes that were expressed at twofold-lower levels in stationary-phase *sarA* mutant cells than in wild-type cells were identified. We rationalized that this decrease in expression within the *sarA* mutant could most likely be due to either (i) a direct loss of SarA activator function or (ii) an indirect consequence of the *sarA* mutation, which decreases production of RNAIII and/or other transcriptional activators and reduces transcription of an *agr*-responsive gene. To distinguish between these two possibilities, transcription profiles (of genes fitting the criteria above) of *agr* and *agr sarA* mutant cells were compared. Genes directly activated by

TABLE 2. *agr*-upregulated genes

ORF[a]	Gene[b]	Pattern[c]	SarA effect[d]	Function[e]	Fold change[f]	N315 ORF[g]	Role category[h]	Sar box[i]
373	*pyrAA*	1 (1)	Up	Carbamoyl-phosphate synthetase	3.3	SA1045	Nucleic acid metabolism	
591		1 (1)	Up	Cytidylate kinase	2.3	SA0515	Nucleic acid metabolism	
3298	*cap5J*	1 (2)	Up	Capsule gene	3.6	SA0153	Adaptation	
831	*dps*	2 (2)	Indirect	General stress protein 20U	3.0	SA1941	Adaptation	
4897	*fmt*	1 (2)	Indirect	Methionyl-tRNA formyltransferase	2.8	SA1059	Aminoacyl tRNA synthetases	
4084	*arcA*	2 (2)	Indirect	Arginine deaminase	5.7	SA2428	Amino acid metabolism	
422	*arcB*	1 (1)	Indirect	Aspartate/ornithine carbamoyltransferase	8.7	SA2427	Amino acid metabolism	
1220	*arcC*	2 (3)	Down	Carbamate kinase	7.9	SA2425	Amino acid metabolism	
1321	*aroC*	1 (1)	Up	Chorismate synthetase	3.1	SA1299	Amino acid metabolism	
5609	*cpsA*	1 (1)	Indirect	Amino acid amidohydrolase	2.6	SA0507	Amino acid metabolism	
3360	*gltB*	1 (2)	Indirect	Glutamate synthase	2.3	SA0431	Amino acid metabolism	
997	*hutG*	1 (1)	Up	Arginase	4.5	SA2125	Amino acid metabolism	Yes
774	*hutH*	2 (3)	Indirect	Histidine ammonia-lyase	4.0	SA0008	Amino acid metabolism	
1001	*hutI*	2 (2)	Indirect	Imidazolone-5-propionate hydrolase	41.5	SA2121	Amino acid metabolism	Upstream
1000	*hutU*	1 (1)	Indirect	Uroconate hydratase	23.3	SA2122	Amino acid metabolism	Yes
4969	*ocd*	1 (1)	Indirect	Ornithine-cyclodecarboxylase	2.5	SA0113	Amino acid metabolism	
2628	*pepF*	2 (3)	Up	Peptidase	3.4	SA1216	Amino acid metabolism	
6	*sdhA*	1 (3)	Indirect	L-Serine deaminase	6.5	SA2318	Amino acid metabolism	
7	*sdhB*	1 (2)	Up	L-Serine dehydratase beta subunit	7.6	SA2319	Amino acid metabolism	
1765	*epiF*	1 (2)	Up	ABC-type multidrug transport system	2.4		Antibiotic production	
4746	*epiP*	1 (2)	Up	Serine protease	2.5		Antibiotic production	
3699		1 (1)	Up	Antibacterial peptide synthesis	5.1	SA0173	Antibiotic production	
1142		1 (2)		Bacteriophage gene	2.3	SA1786	Bacteriophage related	
1132		1 (1)	Indirect	Putative integrase activator	3.6	SAS062	Bacteriophage related	
5455	*femB*	1 (4)		*mec* resistance	2.2	SA1207	Cell wall	
2618		1 (1)		UTP-glucose-1-phosphate uridylyltransferase	2.6		Cell wall	
4659	*moaB*	1 (2)	Indirect	Molybdenum cofactor	2.7	SA2070	Coenzyme metabolism	
3390	*recQ*	1 (6)	Indirect	Probable DNA helicase	2.8	SA0676	DNA replication	
3585	*rocA*	1 (2)	Indirect	NAD-dependent aldehyde dehydrogenases	2.7	SA2341	Electron transport	
4103	*pckA*	1 (2)	Indirect	Phosphoenolpyruvate carboxykinase	3.6	SA1609	Glycolysis	
3898	*crtN*	1 (2)	Up	Putative phytoene dehydrogenase	2.1	SA2351	Lipid metabolism	
882		1 (2)	Indirect	Acyl-coenzyme A dehydrogenase	2.2	SA2080	Lipid metabolism	
1399	*chiB*	1 (1)	Indirect	Predicted chitinase B	2.8	SA0914	Miscellaneous	
1398		1 (1)	Indirect	Predicted chitinase B	2.7	SA0914	Miscellaneous	
5163		1 (3)	Indirect	Putative GTPase	2.7	SA1086	Miscellaneous	
1044		1 (1)	Indirect	Cysteine sulfinate desulfinase	2.9	SA1450	Miscellaneous	
2444	*agrA*	1 (1)	Indirect	*agr* response	5.8	SA1844	Signal transduction	Upstream
1426	*agrB*	1 (1)	Indirect	*agr* response	3.4	SA1842	Signal transduction	Yes
2443	*agrC*	2 (2)	Indirect	*agr* response	4.8	SA1843	Signal transduction	Upstream
1425	*agrD*	1 (1)	Indirect	*agr* response	5.9	SAS066	Signal transduction	Upstream
884	*odhA*	6 (6)	Indirect	2-Oxoglutarate dehydrogenase	2.3	SA1245	TCA cycle	
885	*odhB*	2 (2)	Indirect	2-Oxoglutarate dehydrogenase	3.1	SA1244	TCA cycle	
1221	*arcR*	1 (1)	Down	Transcriptional regulator, Crp/Fnr family	18.9	SA2424	Transcriptional regulation	
4367	*pyrR*	1 (2)	Indirect	Transcriptional attenuator, pyrimidine biosynthesis	2.8	SA1041	Transcriptional regulation	
3806		2 (3)	Indirect	Transcriptional regulator, RpiR family	2.5	SA0187	Transcriptional regulation	Upstream
5259		1 (3)	Up	Transcriptional regulator, GntR family	5.2	SA0434	Transcriptional regulation	
2852		1 (1)	Up	Putative transcriptional regulator, GntR family	2.4	SA1120	Transcriptional regulation	
5005		1 (2)	Indirect	Acetyltransferases	3.0	SA2052	Translation	
421	*arcD*	2 (2)	Indirect	Arginine/ornithine antiporter	6.4	SA2426	Transport	
4309	*fhuD*	1 (2)	Up	Ferrichrome-binding periplasmic proteins	3.2	SA2079	Transport	
5224	*gltP-2*	2 (4)	Indirect	Proton/sodium-glutamate transport	4.3	SA0368	Transport	Yes
2883	*lysP*	1 (1)	Indirect	Lysine-specific permease	2.3	SA1505	Transport	

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TABLE 2—*Continued*

ORF[a]	Gene[b]	Pattern[c]	SarA effect[d]	Function[e]	Fold change[f]	N315 ORF[g]	Role category[h]	Sar box[i]
2606	*opp-1F*	1 (3)	Indirect	Oligopeptide transporter, ATPase domain	2.3	SA2251	Transport	
2601	*opp-2B*	1 (3)	Up	Oligopeptide transporter	2.4	SA1214	Transport	Yes
3147	*potD*	1 (2)	Indirect	Spermidine/putrescine transport protein D	2.3	SA0953	Transport	
2978		1 (1)	Indirect	ABC-type Mn/Zn transporter	2.5	SA0588	Transport	
922		2 (2)	Indirect	ABC-type Mn/Zn transport system component	2.7	SA0589	Transport	
2096	*veg*	1 (1)	Indirect		2.7	SA0452	Unknown	
987		1 (1)	Indirect	Conserved hypothetical	12.6	SA0170	Unknown	
992		1 (3)	Up	Conserved hypothetical	14.3	SA0174	Unknown	
2756		2 (4)	Indirect	Conserved hypothetical	7.0	SA0184	Unknown	Yes
3090		2 (2)	Indirect		2.2	SA0185	Unknown	Upstream
4927		2 (2)		Conserved hypothetical	3.0	SA0271	Unknown	
4370		1 (2)	Up		3.4	SA1037	Unknown	
2479		1 (2)		Conserved hypothetical	7.0	SA1275	Unknown	
2782		1 (2)	Up	Hypothetical protein	2.3	SA1319	Unknown	
2054		1 (1)	Indirect	Predicted membrane protein	2.4	SA1379	Unknown	
2065		1 (2)	Indirect	Conserved hypothetical	3.0	SA1436	Unknown	
2394		1 (1)	Indirect	Conserved hypothetical	2.2	SA1684	Unknown	
2122		1 (2)	Indirect	Conserved hypothetical	3.4	SA2128	Unknown	
1555		1 (2)	Up	Acetyltransferase	2.0	SA2161	Unknown	
9		1 (1)	Up	Hypothetical protein	3.6	SA2321	Unknown	
37		1 (1)	Up	Hypothetical protein	17.3	SA2343	Unknown	
3982		1 (2)	Up	Conserved hypothetical	4.7	SA2491	Unknown	
237		1 (1)	Indirect	Hypothetical protein	2.1	SAS013	Unknown	
644		1 (1)	Up	Hypothetical protein	5.7	SAS016	Unknown	
203		1 (1)	Down	Hypothetical protein	3.2		Unknown	
1494		1 (1)	Indirect	Hypothetical protein	2.3		Unknown	
2275		1 (1)	Up	Hypothetical protein	2.4		Unknown	
2617		1 (2)	Indirect	Hypothetical protein	2.1		Unknown	
2827		1 (1)	Indirect	Hypothetical protein	4.3		Unknown	
2968		1 (1)	Indirect	Hypothetical protein	2.3		Unknown	
5500		1 (3)	Indirect	Hypothetical protein	2.3		Unknown	
3653	*aur*	1 (5)	Indirect	Aureolysin	2.8	SA2430	Virulence factors	Upstream
396	*clfB*	3 (6)	Indirect	Clumping factor B	2.7		Virulence factors	
583	*geh*	2 (2)	Indirect	Lipase (glycerol ester hydrolase)	3.5	SA0309	Virulence factors	
4061	*hla*	1 (1)	Up	Alpha-toxin	38.8	SA1007	Virulence factors	Yes
1427	*hld*	2 (2)	Indirect	Delta-hemolysin	7.9	SAS065	Virulence factors	Yes
1928	*hlgB*	1 (1)	Up	Gamma-hemolysin component B	48.6	SA2209	Virulence factors	Upstream
1927	*hlgC*	1 (1)	Up	Gamma-hemolysin component C	6.3	SA2208	Virulence factors	Yes
667	*lip*	2 (2)		Lipase	3.4	SA2463	Virulence factors	Yes
1175	*pls*	1 (2)	Up	Related to surface protein LPXTG motif	3.3	SA2284	Virulence factors	
2036	*set8*	1 (3)	Indirect	Exotoxin 2	2.2	SA0384	Virulence factors	
2928	*splA*	2 (2)	Indirect	Serine protease	2.5	SA1631	Virulence factors	Yes
2929	*splB*	1 (1)	Up	Serine protease	9.6	SA1630	Virulence factors	Upstream
324	*splD*	2 (2)	Up	Serine protease	ND	SA1628	Virulence factors	Upstream
327	*splF*	2 (2)	Up	Serine protease	11.3	SA1627	Virulence factors	Upstream
2175	*sspC*	1 (1)	Down	Protease	2.4	SA0899	Virulence factors	
2927		1 (10)		Similar to streptococcal adhesin Emb	3.0	SA1268	Virulence factors	
1029		1 (2)	Up	Related to map protein-surface protein	7.7	SA2006	Virulence factors	
4374		2 (2)	Up	Surface protein map	3.1	SA2006	Virulence factors	
5147		3 (15)	Indirect	Homolog of streptococcal hemagglutinin	3.1	SA2447	Virulence factors	
4523		2 (2)	Up	Phenol-soluble modulin beta 2	6.2		Virulence factors	

[a] Designated *S. aureus* GeneChip ORF number.
[b] Previously described gene name.
[c] Many genes are represented on the *S. aureus* GeneChip in a redundant manner as full or partial fragments. Values are the number of times a gene transcript was identified as fitting a pattern (number of times the gene was either partially or completely represented on the GeneChip).
[d] Expected SarA contribution to *agr*-regulated gene transcription.
[e] Previously described gene product function.
[f] Fold change in expression of *agr*-regulated genes as cells transition from the early-log to the stationary phase of growth.
[g] Corresponding designated *S. aureus* strain N315 gene (20).
[h] Expected metabolic role. TCA, tricarboxylic acid.
[i] Yes, putative Sar box identified within the gene's promoter region. Upstream, the gene is expected to be part of an operon with a potential Sar box located in an upstream promoter region.

TABLE 3. *agr*-downregulated genes

ORF[a]	Gene[b]	Pattern[c]	SarA effect[d]	Function[e]	Fold change[f]	N315 ORF[g]	Role category[h]	Sar box[i]
2515	*bsaA*	1 (2)		Glutathione peroxidase	5.8	SA1146	Adaptation	
1889	*ald*	2 (2)		Alanine dehydrogenase	11.9	SA1272	Amino acid metabolism	
5061	*bfmBAB*	1 (2)		Thiamine pyrophosphate-dependent dehydrogenases	26.8	SA1347	Amino acid metabolism	
626	*nasE*	1 (3)		Assimilatory nitrite reductase	115.7	SA2187	Amino acid metabolism	
1888	*tdcB*	2 (2)	Down	Threonine dehydratase	33.8		Amino acid metabolism	
1664	*ddh*	1 (2)		D-specific D-2-hydroxyacid dehydrogenase	2.5	SA2312	Carbohydrate metabolism	
482	*treA*	1 (1)		Alpha-glucosidase	10.8	SA0433	Carbohydrate metabolism	
4571	*folD*	1 (2)		5,10-Methylene-tetrahydrofolate dehydrogenase	6.5	SA0915	Coenzyme metabolism	
2000	*hemL*	1 (4)		Glutamate-1-semialdehyde aminotransferase	10.9	SA1491	Coenzyme metabolism	
625	*sirB*	1 (3)		*S*-adenosyl-L-methionine	ND	SA2186	Coenzyme metabolism	
4186	*thiD*	1 (2)	Down	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	3.7	SA0537	Coenzyme metabolism	
5593	*dinG2*	1 (4)		Putative ATP-dependent DNA helicase	4.7	SA1288	DNA replication	
777	*gyrA*	2 (7)		DNA gyrase	6.2	SA0006	DNA replication	
5293	*mnhA*	1 (7)		Multisubunit Na^+/H^+ antiporter	5.1	SA0813	Electron transport	
2092	*mnhF*	1 (1)		Multisubunit Na^+/H^+ antiporter	11.5	SA0808	Electron transport	
3524	*narG*	2 (9)		Anaerobic dehydrogenases	93.4	SA2185	Electron transport	
622	*narJ*	1 (2)		Nitrate reductase	ND	SA2183	Electron transport	
4539	*hit*	1 (2)		Diadenosine tetraphosphate (Ap4A) hydrolase	2.7	SA1656	Miscellaneous	
1366		1 (2)		Protease related to collagenase	3.0	SA1441	Miscellaneous	
483		1 (3)		Transcriptional regulator, GntR family	6.5	SA0434	Transcriptional regulation	
1505	*pth*	1 (2)		Peptidyl-tRNA hydrolase	2.2	SA0460	Translation elongation	
1732	*dltD*	1 (3)		DttD protein	58.3	SA0796	Transport	
4405	*glpF*	1 (2)	Down	Glycerol uptake facilitator	3.9	SA1140	Transport	
952	*opuD*	1 (2)		Choline-glycine betaine transporter	3.0	SA1183	Transport	Yes
481	*treP*	1 (1)		Phosphotransferase system IIC component	13.1	SA0432	Transport	
4806		2 (4)		Putative efflux membrane protein	18.4	SA1269	Transport	
1887		2 (3)		Amino acid permease	ND	SA1270	Transport	
2206		1 (2)		Putative glucarate transporter	4.6	SA2300	Transport	
121		1 (3)		Conserved hypothetical	ND	SA0212	Unknown	
100		1 (2)	Down	Conserved hypothetical	34.0	SA0412	Unknown	
4122		1 (2)	Down	Conserved hypothetical	11.5	SA2378	Unknown	Yes
4081	*spa*	2 (2)	Down	Protein A	4.4	SA0107	Virulence factors	Yes
3910	*ssaA*	1 (2)		Secretory antigen precursor	17.0	SA2093	Virulence factors	
1941		3 (3)		Myosin cross-reactive antigen	2.8	SA0102	Virulence factors	Yes

[a] Designated *S. aureus* GeneChip ORF number.
[b] Previously described gene name.
[c] Many genes are represented on the *S. aureus* GeneChip in a redundant manner as full or partial fragments. Values are the number of times a gene transcript was identified as fitting a pattern (number of times the gene was either partially or completely represented on the GeneChip).
[d] Expected SarA contribution to *agr*-regulated gene transcription.
[e] Previously described gene product function.
[f] Fold change in expression of *agr*-regulated genes as cells transition from the early-log to the stationary phase of growth. ND, not determined.
[g] Corresponding designated *S. aureus* strain N315 gene (20).
[h] Expected metabolic role.
[i] Yes, putative Sar box identified within the gene's promoter region. Upstream, the gene is expected to be part of an operon with a potential Sar box located in an upstream promoter region.

SarA could be identified as transcripts showing twofold reduction in stationary-phase *agr sarA* double-mutant cells relative to *agr* mutant cells. Several ORFs, including known SarA-activated genes, demonstrated background expression levels in *agr* mutant cells and were considered potentially activated by SarA (Table 4). Additionally, genes transcribed at twofold-higher levels in stationary-phase *agr* mutant cells are listed in Table 4. As expected our analysis identified several known SarA-upregulated virulence genes, including *agrB*, *agrD*, *agrC*, *agrA*, *hld*, and *hla* (6, 8, 13, 15). Additionally, other virulence factors, such as gamma-hemolysin, were determined to be upregulated by SarA. Potential SarA contributions, as defined by the above criteria, to the *agr*-regulated genes are listed in Tables 2 and 3.

Identification of SarA-downregulated genes. SarA-downregulated genes were identified as producing at least twofold-higher transcript levels in stationary-phase *sarA* mutant cells than in wild-type cells. Additionally, genes were considered present in at least one wild-type stationary-phase sample, as determined by GeneChip analysis. Genes with transcript levels meeting these criteria could include (i) genes that are directly repressed by SarA, in which case the absence of SarA allows for increased gene expression, or (ii) genes repressed by RNAIII, whereby decreased *agr* transcription allows derepression of gene expression. To distinguish between these two possibilities, transcript patterns of these genes were further compared in *agr* and *agr sarA* mutant cells. Genes directly repressed by SarA could be identified as producing elevated transcript levels in stationary-phase double-mutant cells compared to *agr* mutant cells (or at background levels). Table 5 lists genes that were determined to be downregulated by SarA in a cell density-dependent manner.

TABLE 4. SarA-upregulated genes

ORF[a]	Gene[b]	Pattern[c]	Function[d]	N315 ORF[e]	Role category[f]	Sar box[g]
3298	*capJ*	1 (2)	Capsule gene	SA0153	Adaptation	
1321	*aroC*	1 (1)	Chorismate synthetase	SA1299	Amino acid metabolism	
997	*hutG*	1 (1)	Arginase	SA2125	Amino acid metabolism	Yes
452	*metK*	1 (2)	*S*-Adenosylmethionine synthetase	SA1608	Amino acid metabolism	
2628	*pepF*	1 (4)	Peptidase	SA1216	Amino acid metabolism	
7	*sdhB*	1 (2)	L-Serine dehydratase	SA2319	Amino acid metabolism	
821		1 (2)	Acetyltransferase	SA1931	Amino acid metabolism	Yes
1765	*epiF*	1 (2)	Epidermin-like biosynthetic cluster		Antibiotic production	
4746	*epiP*	1 (2)	Serine protease		Antibiotic production	
4442		1 (2)	Bacteriophage protein	SA1786	Bacteriophage related	
3688		1 (7)	Predicted membrane protein	SA2436	Bacteriophage related	
1973	*rodA*	1 (3)	Cell division membrane protein	SA1888	Cell division	
184	*femA*	1 (2)	*mec* resistance	SA1206	Cell wall	
2497	*tagF*	1 (2)	Glycosyl/glycerophosphate transferases	SA0244	Cell wall	
3495	*uppS*	1 (4)	Undecaprenyl diphosphate synthase	SA1103	Cell wall	
600		1 (3)	Putative glycosyltransferases	SA0523	Cell wall	
4757		1 (2)	6-Pyruvoyl-tetrahydropterin synthase	SA0666	Coenzyme metabolism	
3095	*dnaG*	1 (2)	DNA primase	SA1391	DNA replication	
3733	BH2391	1 (1)	Short-chain alcohol dehydrogenases	SA1123	Lipid metabolism	
3898	*crtN*	1 (2)	Putative phytoene dehydrogenase	SA2351	Lipid metabolism	
797	*purA*	1 (3)	Adenylosuccinate synthase	SA0016	Nucleic acid metabolism	
373	*pyrAA*	1 (1)	Carbamoyl-phosphate synthetase	SA1045	Nucleic acid metabolism	
783	*rpmH*	1 (1)	Ribosomal protein L34	SAS093	Ribosomal proteins	
1118	*queA*	2 (4)	*S*-Adenosylmethionine	SA1466	RNA modification	
2444	*agrA*	1 (1)	*agr* response	SA1844	Signal transduction	Upstream
1426	*agrB*	1 (1)	*agr* response	SA1842	Signal transduction	Yes
2443	*agrC*	2 (2)	*agr* response	SA1843	Signal transduction	Upstream
1425	*agrD*	1 (1)	*agr* response	SAS066	Signal transduction	Upstream
2944	*phoP*	1 (1)	Response regulator	SA1516	Transcriptional regulation	Yes
5259		1 (3)	Transcriptional regulator, GntR family	SA0434	Transcriptional regulation	
2852		1 (2)	Putative transcriptional regulator	SA1120	Transcriptional regulation	
1545	*prfA*	1 (1)	Protein chain release factor A	SA1920	Translation termination	
4309	*fhuD*	1 (2)	Ferrichrome-binding periplasmic protein	SA2079	Transport	
614	*gltS*	1 (1)	Glutamate permease	SA2135	Transport	
2601	*opp-2B*	1 (4)	Putative oligopeptide transporter	SA1214	Transport	Yes
271		1 (3)	Putative permease	SA0099	Transport	
272		1 (2)	Putative transport	SA0100	Transport	Upstream
4602		1 (2)	Bmr-like protein	SA2241	Transport	
4200		1 (2)		SA0085	Unknown	
992		1 (3)	Conserved hypothetical	SA0174	Unknown	
243		1 (1)	Conserved hypothetical	SA0350	Unknown	
2095		1 (1)	Hypothetical protein	SA0453	Unknown	Yes
2634		1 (3)	Unknown	SA0754	Unknown	
4370		1 (2)		SA1037	Unknown	
890		1 (2)	Conserved hypothetical	SA1240	Unknown	
2479		1 (2)	Conserved hypothetical	SA1275	Unknown	
2782		1 (2)	Hypothetical protein	SA1319	Unknown	
2785		2 (6)	Conserved hypothetical	SA1388	Unknown	
5283		1 (2)	Conserved hypothetical	SA1611	Unknown	
5066		1 (2)	Conserved hypothetical	SA1684	Unknown	
4929		1 (2)	Hypothetical protein	SA1889	Unknown	
5521		1 (2)	Hypothetical protein	SA1928	Unknown	
2505		1 (2)	Hypothetical protein	SA1944	Unknown	
1555		1 (2)	Acetyltransferase	SA2161	Unknown	
9		1 (1)	Hypothetical protein	SA2321	Unknown	
37		1 (1)	Hypothetical protein	SA2343	Unknown	
3982		1 (2)	Conserved hypothetical	SA2491	Unknown	
644		1 (1)	Hypothetical protein	SAS016	Unknown	
2105		1 (2)	Conserved hypothetical		Unknown	
2275		1 (1)	Hypothetical protein		Unknown	
4044	*fnbA*	1 (7)	Fibronectin binding protein A	SA2291	Virulence factors	
3636	*fnbB*	1 (3)	Fibronectin binding protein B	SA2291	Virulence factors	
4061	*hla*	1 (1)	Alpha-toxin	SA1007	Virulence factors	Yes
4578	*hld*	2 (2)	Delta-hemolysin	SAS065	Virulence factors	Yes
1928	*hlgB*	1 (5)	Gamma-hemolysin component B	SA2209	Virulence factors	Upstream
1927	*hlgC*	1 (2)	Gamma-hemolysin component C	SA2208	Virulence factors	Yes
366	*hsa*	1 (X)	Homolog of streptococcal hemagglutinin	SA2447	Virulence factors	
4374	*map*	3 (3)	Map protein	SA2006	Virulence factors	

Continued on following page

TABLE 4—*Continued*

ORF[a]	Gene[b]	Pattern[c]	Function[d]	N315 ORF[e]	Role category[f]	Sar box[g]
1175	*pls*	1 (2)	Related to Pls and accumulation-associated proteins, LPXTG motif	SA2284	Virulence factors	
2343	*sdrc*	2 (2)	Surface protein (SdrC)		Virulence factors	
2037	*set9*	1 (4)	Staphylococcal exotoxin	SA0385	Virulence factors	
5329	*splA*	1 (2)	Serine protease	SA1631	Virulence factors	Yes
2929	*splB*	1 (1)	Serine protease	SA1630	Virulence factors	Upstream
324	*splD*	1 (1)	Serine protease	SA1628	Virulence factors	Upstream
327	*splF*	2 (2)	Serine protease	SA1627	Virulence factors	Upstream
4523		2 (2)	Phenol soluble modulin beta 2		Virulence factors	

[a] Designated *S. aureus* GeneChip ORF number.
[b] Previously described gene name.
[c] Many genes are represented on the *S. aureus* GeneChip in a redundant manner as full or partial fragments. Values are the number of times a gene transcript was identified as fitting a pattern (number of times the gene was either partially or completely represented on the GeneChip).
[d] Previously described gene product function.
[e] Corresponding designated *S. aureus* strain N315 gene (20).
[f] Expected metabolic role.
[g] Yes, putative Sar box identified within the gene's promoter region. Upstream, the gene is expected to be part of an operon with a potential Sar box located in an upstream promoter region.

DISCUSSION

Using oligonucleotide microarray technology for genome-wide transcription profiling, we have identified *S. aureus* genes with expression patterns expected of genes either up- or down-regulated in a cell-density *agr*- and/or SarA-dependent manner. Given that most known *S. aureus* virulence factors are temporally regulated by one, if not both, of these effectors, it is likely that the genes identified within this study include previously unrecognized virulence determinants.

Although investigators have successfully used transcription profiling technology to study biological processes in a number of other organisms, we initially set out to validate the methodology used by determining whether the results obtained with the *S. aureus* GeneChip correlated with our Northern blot data and previous reports. As expected, profile analysis of the effector of the *agr* response (RNAIII) demonstrated that transcript titers increased in a cell density-dependent manner within wild-type cells but were undetectable in *agr* mutant strains. Results also confirmed that RNAIII expression is diminished in a *sarA* mutant, further establishing that SarA is required for wild-type levels of RNAIII production or that the absence of SarA delays the onset of RNAIII expression. Likewise, the transcription profiles of alpha-toxin produced results that are consistent with other reports and confirmed that it is upregulated by each effector. Interestingly, profiling indicated that *hla* expression in RN6911 cells increased 2.2-fold in a growth phase-dependent manner. This increase was not detected by Northern analysis (Fig. 2B) but has also been reported by others (6), suggesting that the GeneChip technology used may be more reliable than Northern blot analysis. Profiling data for protein A also supported both previous reports, as well as our Northern blot data, and confirmed that genes that are downregulated by each effector can be detected in our system. The protein A transcription profiles indicate that the observed derepression of *spa* transcription occurs throughout all growth phases of *agr* and *sarA* mutant cells, implying that either RNAIII or SarA production may repress expression of an activator of protein A in wild-type cells. Indeed, it has recently been found that either *agr* or *sarA* mutant cells produce elevated amounts of SarS (also known as SarHI), an activator of *spa* transcription (11, 33). More specifically, in those studies it was shown that *sarA* cells produced more *sarS* transcripts than did *agr* mutant cells, which nicely correlates with the *spa* transcript patterns observed here. In fact, our transcription profiling results indicate that (i) *sarS* is constitutively expressed at low levels in wild-type cells, (ii) the gene is transcribed above wild-type levels in both RN6911 and ALC842 cells during early growth phases, but transcript levels decrease to wild-type amounts at later growth phases, and (iii) SarS is constitutively expressed at levels 10-fold higher than wild-type levels in *sarA* cells (data not shown). These results suggest that the mechanisms of *sarS* derepression differ for *sarA* and *agr* cells. This has subsequently been confirmed by additional transcription profiling studies (unpublished data). Results of the present study also demonstrated that the profiles of at least 10 additional genes correlated with the work of other groups, including *splA*, *splB*, *splC*, and *splD* (31); *sarS* (11); *agrA*, *agrB*, *agrC*, and *agrD* (RNAII [7, 13, 19, 27]); and *fnbA* (35). Collectively these results provide a strong indication that the transcription profiling procedure used correlates well with previous *agr* and/or *sarA* studies, and they verify the methodology.

In all, 104 genes were revealed to be upregulated in a cell density- and *agr*-dependent manner. Among these genes (Table 2) were 20 putative virulence determinants, including 14 genes involved in extracellular factor production. Because their expression corresponds directly with *agr* transcription, presumably these genes were induced directly in response to RNAIII. In contrast, 34 genes appeared to be downregulated in an *agr*-dependent manner (Table 3). Two of these genes, *spa* (protein A) and ORF1941 (cross-reactive antigen), encode putative cell surface virulence factors. One potential extracellular virulence factor, *sspA* (secretory antigen precursor), was found to be downregulated by *agr*.

There appears to be a trend in the expression of *agr*-regulated virulence factors. Collectively our results suggest that an *agr* determinant upregulates extracellular virulence factors but downregulates cell surface virulence factors. Although this has long been a common hypothesis among investigators, the present body of work provides an unprecedented corrobora-

TABLE 5. SarA-downregulated genes

ORF[a]	Gene[b]	Pattern[c]	Function[d]	N315 ORF[e]	Role category[f]	Sar box[g]
2462	*clpL*	2 (2)	ATPase/chaperone	SA2336	Adaptation	
1220	*arcC*	2 (2)	Carbamate kinase	SA2425	Amino acid metabolism	
1191	*gudB*	1 (2)	Glutamate dehydrogenase/leucine dehydrogenase	SA0819	Amino acid metabolism	Upstream
4470	*rocD*	1 (3)	Ornithine aminotransferase	SA0818	Amino acid metabolism	Yes
1075		2 (2)	Proline dehydrogenase	SA1585	Amino acid metabolism	
1888		1 (2)	Threonine dehydratase		Amino acid metabolism	
2369	*aldA*	1 (3)	NAD-dependent aldehyde dehydrogenases	SA0162	Carbohydrate metabolism	
4567	*fhs*	1 (5)	Formyl-tetrahydrofolate synthetase	SA1553	Carbohydrate metabolism	
1416		2 (3)	Dihydroxyacetone kinase	SA0605	Carbohydrate metabolism	
5238	*atl*	1 (5)	Autolysin	SA0905	Cell division	
2381	*pbp3*	1 (2)	Penicillin-binding protein 3	SA1381	Cell wall	
4186	*thiD*	1 (2)	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	SA0537	Coenzyme metabolism	
2912		1 (3)	Homology to *N*-carbamoylsarcosine amidohydrolase	SA2438	Coenzyme metabolism	
4407	*mutL*	1 (4)	DNA mismatch repair protein	SA1138	DNA repair	
3933	*ndhF*	1 (3)	NADH-ubiquinone oxidoreductase subunit 5	SA0411	Electron transport	
35	*rocA*	3 (4)	1-Pyrroline-5-carboxylate dehydrogenase	SA2341	Electron transport	
4603		1 (3)	Carboxylesterase type B	SA2240	Lipid metabolism	
123	*IrgB*	1 (2)	Holin-like protein	SA0253	Miscellaneous	
2047	*purM*	1 (3)	Phosphoribosylaminoimidazol synthetase	SA0923	Nucleotide and nucleic acid metabolism	
1996	*citC*	1 (1)	Isocitrate dehydrogenase	SA1517	TCA cycle	
265		1 (6)	Putative transcription antiterminator, BgIG family	SA1961	Transcription termination	
1221	*arcR*	1 (1)	Transcriptional regulator, Crp/Fnr family	SA2424	Transcriptional regulation	
3985		1 (3)	Transcriptional regulator, RpiR family	SA0187	Transcriptional regulation	Upstream
4405	*glpF*	1 (2)	Glycerol uptake facilitator	SA1140	Transport	
2839	*gntP*	1 (4)	Gluconate permease	SA2293	Transport	
205		1 (2)	Phosphotransferase system IIB components	SA0186	Transport	Upstream
4259		1 (2)		SA0021	Unknown	
3420		1 (3)	Conserved hypothetical	SA0212	Unknown	
2103		1 (2)	Conserved hypothetical	SA0271	Unknown	
2424		1 (2)		SA0363	Unknown	
100		1 (2)	Conserved hypothetical	SA0412	Unknown	
2823		2 (3)	Conserved hypothetical	SA1618	Unknown	
827		1 (1)	Conserved hypothetical	SA1937	Unknown	
4122		1 (1)	Conserved hypothetical	SA2378	Unknown	Yes
367		1 (2)	Conserved hypothetical	SA2448	Unknown	
203		1 (1)	Hypothetical protein		Unknown	
2577		1 (2)	Hypothetical protein		Unknown	
426	*aur*	1 (5)	Aureolysin	SA2430	Virulence factors	Upstream
427	*isaB*	1 (2)	Immunodominant antigen B	SA2431	Virulence factors	Yes
4197	*lip*	1 (2)	Lipase	SA2463	Virulence factors	Yes
2574	*nuc*	1 (1)	Nuclease	SA0746	Virulence factors	Yes
4081	*spa*	2 (2)	Protein A	SA0107	Virulence factors	Yes
2174	*sspB*	1 (1)	Cysteine protease precursor	SA0900	Virulence factors	
2175	*sspC*	1 (1)	Protease	SA0899	Virulence factors	

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[e] Corresponding designated *S. aureus* strain N315 gene (20).
[f] Expected metabolic role. TCA, tricarboxylic acid.
[g] Yes, putative Sar box identified within the gene's promoter region. Upstream, the gene is expected to be part of an operon with a potential Sar box located in an upstream promoter region.

tion of this proposal. An immediate question that remains unanswered is why cell surface virulence factors are expressed during early phases of growth whereas extracellular proteins are produced at higher cell densities.

Traditionally, it has been hypothesized that during early stages of host invasion staphylococci produce MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and other cell surface proteins, which promote attachment to both host cell and foreign (i.e., catheter) surfaces as well as avoidance of host defensive machinery. Once the organism has reached a critical threshold number, virulence factor expression switches to a more invasive system in which the organism produces extracellular proteins capable of degrading host cells, their products (such as antibacterial agents), or their infrastructure (such as phagocytes). This is an attractive yet, based on our SarA findings, potentially oversimplified hypothesis.

As shown in Tables 4 and 5, 76 genes were found to be upregulated in a cell density- and SarA-dependent manner. Among these genes were 16 putative virulence factors, including 10 extracellular and 6 cell surface virulence determinants. Several known SarA-upregulated genes, such as *hla* and *fnbA*,

are included in this list (6, 12, 35). A total of 44 genes, including 5 extracellular and 3 cell surface virulence factors, were found to be downregulated in a SarA-dependent manner. These results suggest that SarA-regulated virulence genes do not necessarily follow the reported reciprocal expression patterns that have been established between *agr*-regulated cell surface and extracellular proteins. Several of the genes identified as being regulated by SarA have been found to harbor putative Sar boxes within their promoter regions, as indicated in each table.

Interestingly, Cheung and colleagues have previously demonstrated that *fnbA* is regulated by SarA (35). Although attempts were made in that study, no *fnbB* signal was detected in Northern blot analysis. Conversely, our profiling results suggest that both *fnbA* and *fnbB* are expressed in a SarA-dependent manner and indicate that GeneChip technology is more sensitive than Northern blot analysis, in that instance. Similarly, numerous attempts to study the transcription of components of both the arginine deiminase and histidine utilization operons (both of which were identified as potentially being regulated by *agr* in our transcription profiling studies) by Northern analysis did not demonstrate any signal, further illustrating the sensitivity of the GeneChip technology.

Many of the genes identified as being regulated by *agr* and/or SarA encode putative virulence factors, yet the majority do not. Although a number of other investigators have proposed that each effector is likely to regulate "nonvirulence genes," data substantiating this hypothesis are sparse. It is likely that because the GeneChip technology used appears to provide far more sensitivity in studying biological processes than conventional approaches, others have not identified many of the genes presented in the present study as being regulated by each effector. Among genes not previously known to be stimulated in an *agr*- and cell density-dependent manner were genes encoding the members (*arcA*, *arcB*, *arcC*, and *arcD*) and the activator (*arcR*) of the arginine deiminase pathway. Activation of this pathway allows *Bacillus licheniformis* to grow anaerobically in the presence of arginine (24), an ability that could be beneficial to *S. aureus* during pathogenesis. Additionally, utilization of arginine via the arginine deiminase pathway produces ammonia, which has been shown to protect bacteria from the deleterious effects associated with acidic environments (4). However, further in vivo studies are required to determine whether components of the arginine deiminase pathway contribute to *S. aureus* pathogenesis.

Members of the histidine utilization pathway (*hutG*, *hutH*, *hutI*, and *hutU*), which constitute a single transcript in *Bacillus subtilis* that is induced primarily by the presence of L-histidine, also appeared to be expressed in an *agr*- and cell density-dependent manner (14). It is difficult to resolve how transcription of this operon contributes directly to *S. aureus* pathogenesis. Given the number of genes upregulated by each effector that are involved in amino acid metabolism and transport pathways, it is tempting to speculate that each regulator not only mediates virulence factor production but may also poise the cell to scavenge for nutrients. Another alternative is that proteases that are directly produced in response to *agr* and/or SarA degrade secreted *S. aureus* factors and activate transport and metabolic processes; as a consequence, these processes may be indirectly inducible by each effector in laboratory cultures. Moreover, it is likely that unrecognized *agr*- and/or SarA-mediated processes that occur during mid-log-phase growth perturb the cell and affect biological processes, which in turn mask the relevance of biological processes determined to be regulated by each effector in the present body of work.

A number of genes identified as regulated by either *agr* or SarA or both have not been previously characterized. It is likely that some, if not many, of these genes contribute to *S. aureus* pathogenesis. Moreover, understanding the functions of these genes may further clarify the biological processes identified here as being stimulated by either effector. Finally, it is important to recognize that pathogenesis is not likely to be a static process; rather, the invading bacterium must cope with fluxes in its immediate environment, such as changes in pH, changes in nutrient levels, differences in cell densities, and interactions with host factors. Therefore, the work presented here should be considered a snapshot of gene expression and a cataloging of the network of genes that are expected to be regulated by each effector in a cell density-dependent manner. We are actively characterizing networks of genes that are regulated by other elements, such as host factors, in an effort to better understand the pathogenic processes of staphylococci.

Because this technology provides transcriptional profiles for each gene tiled onto a gene chip, yet analysis of each individual gene is beyond the scope of this report, we have elected to provide this data to the scientific community, so that others may analyze genes involved in other biological processes. The transcriptional profiling data obtained in these studies can be accessed at the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) website (http//narsaweb.narsa.com).

ACKNOWLEDGMENTS

We thank Ambrose Cheung for providing strains used in this study and Lefa Alksne for critical reading of the manuscript. In addition, we thank the editors of the NARSA website for accommodating our data.

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